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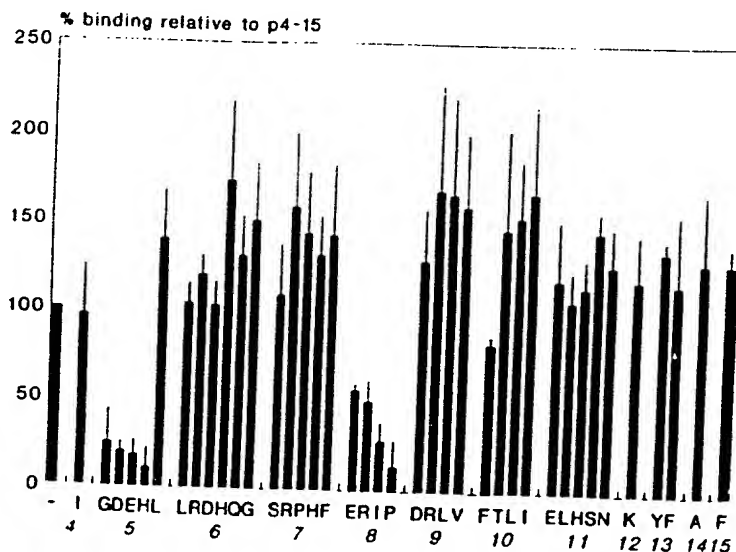
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(54) Title: HLA-DR3 BLOCKING PEPTIDES AND THEIR USE IN THE TREATMENT OF HLA-DR3 ASSOCIATED AUTOIMMUNE DISEASES.



(57) Abstract

A peptide comprising the amino acid sequence: $A_{n-1} A_n A_{n+1} A_{n+2} A_{n+3} A_{n+4} A_{n+5}$ wherein A_{n-1} is any amino acid; A_n is I, L or V; A_{n+1} is A, H or Q; A_{n+2} is Y, S, R or P; A_{n+3} is D, E or Q; A_{n+4} is E or D; A_{n+5} is any amino acid; with the proviso that said sequence differs by one amino acid substitution from the sequence T I A Y D E E. Use of the peptide which has an HLA DR3 blocking activity in a treatment of HLA DR3 related autoimmune diseases.

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HLA-DR3 blocking peptides and their use in the treatment of HLA-DR3 associated autoimmune diseases

FIELD OF THE INVENTION

The invention is concerned with the selection of peptides that can be used in a medical treatment of autoimmune diseases, in particular autoimmune diseases which are associated with human leukocyte antigens (HLA) of type HLA-DR3, such as type I diabetes, Grave's disease, Sjögren's syndrome and myasthenia gravis.

BACKGROUND OF THE INVENTION

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The human immune system can cause disease not only by producing too many antibodies, as in the case of allergies, but also by the production of T cells against healthy cells of the own body. Such T cells reactive with "self"-molecules can cause autoimmune diseases.

15 At the moment there are about 40 diseases known that are suggested to be caused by autoimmune mechanisms, of which the cause and seriousness vary a lot. Furthermore, it has been proven that the occurrence of autoimmune diseases is associated with the presence of certain tissue antigens, the human leukocyte antigens (HLA). Many autoimmune diseases (e.g. type I diabetes, Grave's disease, Sjögren's syndrome, myasthenia gravis) are associated with one particular HLA-antigen, namely HLA-DR3.

20 HLA antigens/molecules are encoded by 6 extremely polymorphic closely linked genes on chromosome 6. There are 3 genes (A,B,C) coding for so-called class I molecules, which are present on the surface of all nucleated cells and can bind and present e.g. virus derived antigenic peptides to CD8⁺ (cytotoxic) T lymphocytes, which then can kill e.g. virus infected cells. Three other genes (DR, DQ, DP) code for so-called class II molecules, HLA-DR being the most important. HLA-DR molecules are tissue antigens that are normally present on professional antigen cells, such as B cells

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and macrophages and that can bind and present peptides from proteins or microbes taken up by those cells to CD4⁺ helper T cells, which in turn can activate other lymphocytes. Each helper T cell can only recognize antigen (peptide) presented by one of the two different HLA-DR molecules present in most individuals. The terminology also used to describe this phenomenon is that each T cell response is restricted by a particular HLA-DR molecule or antigen.

In case of a virus infection or an inflammation when cytokines such as interferon-gamma are produced, DR-antigens can also appear on other body cells. These cells then also become capable to present antigen to T helper cells. This can result in the production of T cells or antibodies directed against these cells of the own body.

The helper T cell, which is a class II restricted CD4 positive T cell, plays a central role in orchestrating the immune response. It produces cytokines or lymphokines which regulate at least all the other antigen-activated components of the immune system. Thus the most specific and efficient immunotherapy for an autoimmune disease would prevent that auto-reactive helper T cells are turned on, i.e. prevent that HLA molecules present an auto-antigen to the T cell receptor of an auto-reactive helper T cell.

A possible strategy would be to prevent the HLA molecule from binding and presenting an auto-antigen that induces an autoimmune disease. Particularly if certain HLA alleles are exclusively or preferentially presenting such an auto-antigen, this can be done in at least two ways; one is the use of antibodies against the HLA molecule or more specifically against a particular combination of peptide and HLA, and the second is the development of peptides or other compounds that prevent the disease inducing epitope from binding to the disease related HLA molecule.

If we would know the disease inducing antigens, we could try to induce tolerance, for instance by delivering that antigen in such a way that it does not induce a detrimental immune response but instead turns off the immune response to that antigen.

Particularly if certain T cell receptors are preferentially used to recognize a particular peptide-HLA combination, we could use antibodies or even better active strategies like vaccination with attenuated disease inducing T cells or T cell receptor peptides.

Another strategy, which at first glance seems to be less specific, uses an antibody directed against the CD4 molecule used by the helper T cell to recognize the HLA class II molecule.

Because for most auto-immune diseases the auto-antigen is not known and no information is available that would permit T cell receptor directed interventions, we are left with the presenting HLA class II molecule to target immune interventions. It might be possible to prevent the activation of self-reactive T helper cells which lead to the destruction of "self" cells and tissues by adding a peptide that binds to a particular HLA-DR molecule but is not recognized by self-reactive helper T cells. Such a peptide, regardless whether it inhibits binding of the self antigen to that HLA-DR molecule or interferes by a different mechanism with the induction of disease by self antigens, is called herein a blocking or competitor peptide. To be useful as an immune modulatory drug, a HLA class II blocking peptide should not be immunogenic (i.e. not activate T cells) and preferably bind selectively to a certain DR type (allele).

SUMMARY OF THE INVENTION

According to our invention DR3-restricted helper T cell responses are blocked in an allele (DR3) specific way with the use of peptides derived by single amino acid substitutions from a DR3-restricted immunodominant peptide epitope of the 65kD heat shock protein of Mycobacteria hsp65 p4-15.

The doses of blocking peptide needed to obtain this effect suggest that this invention may be applied in vivo to block DR3-restricted self-reactive helper T cells and thus prevent and/or treat autoimmune diseases in which DR3-restricted T cells play an important role. Because this immunosuppression is DR3-specific,

helper T cells restricted by other DR alleles and other class II molecules (DQ, DP) will not be affected. Therefore this immune intervention will not result in general immune suppression.

The present invention provides a peptide comprising the amino acid sequence

A_{n-1} A_n A_{n+1} A_{n+2} A_{n+3} A_{n+4} A_{n+5}

wherein

A_{n-1} is any amino acid;

A_n is I, L or V;

10 A_{n+1} is A, H or Q;

A_{n+2} is Y, S, R or P;

A_{n+3} is D, E or Q;

A_{n+4} is E or D;

A_{n+5} is any amino acid;

15 with the proviso that said sequence differs by one amino acid substitution from the sequence T I A Y D E E. Amino acids are identified herein by the one-letter code, i.e. T refers to threonine (Thr), I to isoleucine (Ile), A to alanine (Ala), Y to tyrosine (Tyr), D to aspartic acid (Asp), E to glutamic acid
20 (Glu), etc.

In preferred embodiments of the invention, A_{n-1} is T, A_{n+5} is E, A_n is I, and/or A_{n+3} is D. More preferably the invention relates to a peptide wherein

A_{n-1} is T;

25 A_n is I;

A_{n+1} is A, H or Q;

A_{n+2} is Y, S, R or P;

A_{n+3} is D;

A_{n+4} is E or D;

30 A_{n+5} is E,

with the proviso that A_{n+1} is not A when A_{n+2} is Y and A_{n+4} is E.

Specific examples of preferred embodiments of the invention are peptides comprising an amino acid sequence selected from the group consisting of

35 T I A Y D D E (SEQ ID NO:1)

T I A S D E E (SEQ ID NO:2)

T I A R D E E (SEQ ID NO:3)

T I A P D E E (SEQ ID NO:4)

T I H Y D E E (SEQ ID NO:5)

T I Q Y D E E (SEQ ID NO:6)

5 The length of peptides according to the invention may vary between broad limits, but will vary practically from about 7 to about 30 amino acids. Preferably, the peptide has a length of from about 8 to about 20 amino acids.

10 In the peptide, the amino group of the amino-terminal amino acid may be modified, e.g. acylated. Independent therefrom, also the carboxy group of the carboxy-terminal amino acid may be modified, e.g. amidated.

15 The invention also provides a pharmaceutical composition comprising a HLA DR3 blocking effective amount of a peptide according to the invention as defined above and a pharmaceutically acceptable carrier or diluent.

20 Further, the invention also provides a method of treating a HLA DR3 related autoimmune disease, comprising administering a HLA DR3 blocking effective amount of a peptide according to the invention as defined above to a person or mammal suffering from said HLA DR3 related autoimmune disease.

BRIEF DESCRIPTION OF THE DRAWING

25 Figure 1. Binding to HLA-DR17 BLCL of biotinylated, single substituted analogs of hsp65 p4-15. The position of substitution and the amino acid residue that is substituted (single letter code) are indicated on the x-axis. The percentage of binding relative to the native peptide p4-15 was calculated by dividing
30 the mean fluorescence, corrected for background fluorescence, of each peptide by the mean fluorescence of p4-15 and is indicated on the y-axis. The results represent the mean of three independent experiments.

35 Figure 2A. Inhibition of hsp65 p4-15 stimulated activation of DR3-restricted T cell clones by selected single amino acid substituted analogs of p4-15. The amount of competitor peptide

added is indicated in fold-excess on the x-axis. Proliferation is expressed in cpm on the y-axis. SI for competitor peptides without p4-15 were ≤ 1 . Results were reproducible in several independent experiments.

5 Figure 2B. Inhibition of 30/31kD dependent activation of DR3-restricted T cell clones by selected single amino acid substituted analogs of p4-15 (see legend Fig.2A).

10 Figure 2C. Lack of inhibition of hsp65 p418-427 specific DR2-restricted T cell clone R2F10 and of hsp65 p412-425 specific DR1-restricted T cell clone N3A9 by selected substituted peptides (see legend Fig.2A).

15 Figure 3. Lack of inhibition by other DR3-restricted epitopes (Table II) of the p3-13 induced activation of CAAP15 1-1 (see legend Fig.2A). As a control for positive inhibition the p4-15 substituted analog (7Y→S) was included in the same experiment.

DETAILED DESCRIPTION OF THE INVENTION

20 Antigen recognition by CD4⁺ T cells involves the formation of a trimolecular complex between the TCR, MHC class II molecule and processed antigen (1-3). Processed antigen consists of peptidic antigen fragments that can be represented by linear synthetic peptides (4, 5). To analyze MHC-peptide-TCR interactions, several different approaches (6-8, 27-32) have been used, that all shared
25 a common aspect in that they used single amino acid substituted peptides to monitor the function of each residue in the peptide. Thus, individual amino acid residues in an immunogenic peptide can be defined either as TCR (epitope) or MHC (agretope) contact residues or spacer residues.

30 In order to design competitor peptides that are able to inhibit DR3-restricted responses in an allele specific manner, a better knowledge of those residues involved in contacting DR17 and/or the T cell receptor is necessary. To this end, the peptide
35 T I A Y D E E A R R G L (p4-15) derived from the mycobacterial 65kDa heatshock protein (hsp 65) was selected as a model peptide.

In previous studies it was found that the hsp65 peptide p3-13 is immunodominant in the mycobacterium specific T cell response of HLA-DR3⁺ individuals. The peptide is not recognized in the context of any other HLA-DR molecule (9), most probably because it binds specifically to HLA-DR17(3) molecules (10) only. We have now further examined the interactions between HLA-DR17, p3-13 and several TCRs by using the single amino acid substitution strategy to determine agretope and epitope residues in p3-13. We prepared p3-13 analogs that bound to HLA-DR17 but had lost the capacity to stimulate p3-13 reactive T cell clones and tested their capacity to inhibit the proliferation of p3-13 reactive clones and other HLA-DR17 restricted T cells to respectively p3-13 and other antigens presented by HLA-DR17.

An object of our research was to define synthetic peptides that will compete for recognition of (self)-antigens at the level of DR3-restricted antigen presentation. DR3 specific competitor peptides may be of interest given the association of HLA-DR3 with a considerable number of autoimmune diseases (15).

The interactions between hsp65 p4-15, HLA-DR17(3) and four different TCRs have been analyzed. We have found, using a set of single amino acid substituted N-terminal biotinylated analogs of p4-15 that amino acids I at position 5 and D at position 8 are critical binding residues (Fig.1). Comparison of the sequence of other DR17 binding peptides with that of hsp65 p4-15 has revealed a putative DR17 binding motif. Recently a second 65kD GroEL protein of *M. leprae* has been identified (26) that binds to DR17 (A. Geluk et al. unpublished results) and contains the amino acids I and D at position 5 and 8 respectively. Moreover, the sequence of another known T cell epitope, tetanus toxoid p1273-1284 (25), which is DR52a-restricted, contained the same amino acid residues equally spaced from each other by two amino acids. The DR17 specific peptide binding pocket is preserved in DR52a molecules as the residues of the hypervariable region of DRB3 molecules in DR52a, that are supposed to form the peptide binding groove (10), are identical to those of DRB1 molecules of DR17. Furthermore, analysis of two newly defined DR3-restricted epitopes, from the

hsp70, hsp18 and the 30/31kD protein of *M. leprae* showed that these peptides, except one epitope from the 30/31kD protein, contained a similar DR17-binding motif: the hsp70 derived epitope contained the amino acid L separated by two residues from the amino acid D, whereas in the 18kD-derived peptide position 64 was occupied by V followed by E in position 67. As the amino acids L and V are comparable in hydrophobicity and bulkiness to I and as the amino acid E, like D, contains a negatively charged side chain, we have identified a putative DR17-specific binding motif (Table I). This motif is composed of a large, hydrophobic residue (I, L, V) at position n, followed by a negatively charged (D, E) or polar (Q) residue at position n+3. The fact that a negative charge at position n+3 is critical for peptide-DR17 binding is consistent with our finding that the peptide binding groove of DR17 molecules contains a positively charged pocket, specific for HLA-DR17 molecules (10). Furthermore, residue n (I, L, V) should be flanked by one or more amino acids on its N-terminal side (data not shown) and probably cannot be situated next to the positively charged N-terminus of the peptide. However, only one of the 30/31kD epitopes which are recognized by the two 30/31kD reactive, DR3-restricted clones L10B4 and L10C11 (Thole et al. in preparation) contains the DR17 motif: p62-71 contains I at position n and the polar amino acid Q at position n+3, while the other epitope, p138-146, contains E at position n+3 but no hydrophobic amino acid at position n (Thole et al. in preparation). Thus this DR17 binding motif is present in 6 out of 7 DR3-restricted epitopes.

It has been shown that antigens, the response to which was restricted by the same MHC class II molecule, compete with each other for presentation to T cells (36) and that competition for antigen presentation takes place at the level of MHC class II molecule (37, 38). Since MHC molecules contain a single peptide binding site, as indicated by X-ray crystallography (39) and peptide competition studies (40) it might be feasible to design peptides that bind to MHC molecules but do not activate disease-causing T cells. Such peptides would then act as antagonists for

recognition of self-antigens. The observation that a mouse lysozyme self peptide, itself not immunogenic, can compete for MHC binding with an immunogenic peptide from hen egg-white lysozyme and so reduces T cell activation by that peptide (41), is compatible with such a notion. It has also been shown that allorecognition could be inhibited by peptide competitors for both class I (42) and class II (43, 44) reactive T cells.

We have now shown that the activation of DR3-restricted, hsp65-reactive T cell clones by the hsp65 p4-15 can be inhibited in an allele (DR3) specific manner by peptides that differ only one amino acid from p4-15 in their sequence (Fig.2A). These peptides contain substitutions at either residue 6 (A), 7 (Y) or 9 (E), critical TCR-recognition residues, whereas the residues important for binding to DR17, residues 5 (I) and 8 (D) are unchanged. We have further demonstrated that the competitor peptides are also able to inhibit the activation of two other hsp65 non-reactive, DR3-restricted T cell clones (Fig.2B). The possibility to inhibit the response of these 30/31kD reactive clones with single amino acid substituted analogs of hsp65 p4-15 shows that the inhibition does not, thus far, depend on either the stimulator peptides or the stimulated T cell. Moreover, as we have shown that these competitor peptides do not inhibit the response of a DR1-restricted or a DR2-restricted T cell clone (Fig.2C), inhibition is allele specific.

The mechanism of this allele specific inhibition of T cell proliferation by p4-15 analogs has not yet been investigated by us. On the one hand MHC blockade and/or competition (41) is suggested by the observation that the p4-15 analogs were able to inhibit both the response to the related p4-15 and to the unrelated 30/31kD derived peptide.

However, other mechanisms such as TCR antagonism (45) or tolerance induction (46) can certainly not be ruled out at this stage, also because preliminary results (Fig.3) indicate that DR3 restricted epitopes, other than p4-15 analogs, containing the DR17-peptide binding motif are not able to inhibit the proliferation of p4-15 reactive T cells.

Our results encourage further research aiming at the design of peptides that inhibit (auto)immune responses in an allele specific manner without affecting T cell responses restricted to other alleles. Differences in (self) peptide presentation may be the molecular basis for HLA class II (DR) disease associations and this may offer possibilities for specific immunotherapy with competitor peptides. An example in mice is experimental allergic encephalomyelitis (EAE). Here, allele specific synthetic competitor peptides for disease inducing myelin basic protein epitopes could be used effectively as specific inhibitors of EAE (46). Immunotherapy with DR17 specific competitor peptides may be of interest given the association of HLA-DR17 (DR3) with a considerable number of autoimmune diseases (15).

The most straightforward explanation of this allele specific immune suppression is blocking by the competitor peptides of (newly synthesized) DR3 molecules which are then not available for a presentation of disease inducing auto-antigens in high enough concentration to activate self-reactive DR3-restricted T cells. Antagonism of TCR from such cells might increase the specificity and effectiveness of such a treatment. If this would be the mechanism, long-term and frequent (e.g. daily) administration of DR3-binding competitor peptides might be necessary to achieve and maintain down-regulation of auto-reactive T cells. Although from a pharmacological point of view this has advantages, a treatment of shorter duration would at least be cheaper. The evidence (from experimental animal models and using other peptides) that also other mechanisms than competition for binding to HLA-class II molecules may be involved, e.g. that inhibitory peptides may induce tolerance of auto-reactive helper T cells, suggests that not only passive but also active immunomodulation might be achieved by MHC-binding peptides. In that case short-term courses of limited duration might also be effective. In both cases such immunomodulatory peptides would have to be administered in such a way that effective concentrations can be obtained in the antigen presenting cells presenting the disease inducing auto-antigen. Animal models suggest that systemic administration is a realistic

possibility and organ-specific targeting might increase the effectiveness of such an immunotherapy.

EXAMPLES

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Synthetic peptides

A set of 240 single amino acid substituted analogs of hsp65 p4-15 (each residue substituted by all 20 amino acids including the natively occurring amino acid) was synthesized using the Pepsan (16) method. All other peptides were made on an ABIMED 422 synthesiser (ABIMED, Langenfeld, Germany) using the simultaneous multiple peptide synthesis method (17). N- and C-terminal truncated peptides of hsp65 p2-13 were also synthesized in their N-terminal biotinylated form. These long-chain biotinylated analogs of the peptides were made by coupling of 6-(Fmoc-amino)hexanoic acid and biotin (Serva, Heidelberg, Germany) respectively, at the end of the synthesis (10). All peptides were made as C-terminal amides.

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Cells

HLA-DR homozygous EBV-BLCL used in the binding experiments were obtained from the Xth International Histocompatibility Workshop panel (unless otherwise indicated), and were named: HAR (A1, B8, Cw7, DR17(3), DR52a, Dw3, DQw2, DPw4), AVL (A1, B8, Cw7, DR17(3), DR52a, Dw3, DQw2, DPw4), CAA (A1, B8, Cw7, DR17(3), DR52a, Dw3, DQw2, DPw4), RSH (DR18(3), DR52a, Dw3), OOS (DR1, Dw1), IWB (DR15(2), Dw2), BSM (DR4, DR53, Dw4), ATH (DR11(5), DR52b, Dw5), ABO (DR14(6), DR52b, Dw9), EKR (DR7, DR53, Dw7), MADURA (DR8, DR52, Dw8.1), DKB (DR9, DR53, Dw23). The class II negative B cell line RJ2.2.5 (DR-negative, DQ-negative, DP-low) was kindly provided by Dr. J. B. Rothbard.

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Binding assay

In the binding assay (18) EBV-B lymphoblastoid cell lines (BLCL, 3×10^5 /sample) were incubated with the biotinylated peptide (50 μ M) at 37°C for 20 h. As a control, cells were labelled in each experiment with a biotinylated monoclonal antibody specific for

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HLA-DR (5 μ l; Becton Dickinson, CA) at 4°C for one hour. Peptide or anti-DR preincubation were followed by labelling with FITC-avidin D (10 μ g/ml; 100 μ l; Vector Labs, CA) at 4°C for 30 min. When greater sensitivity was required, the incubation with FITC-avidin D was followed by incubation of biotinylated anti-avidine D (10 μ g/ml; 100 μ l; Vector Labs, CA) and again FITC-avidin D. After each incubation excess reagents were washed off at 4°C using PBS containing 0.1% BSA. Stained cells were analyzed by flowcytometry on a FACScan analyser (Becton-Dickinson, CA). Dead cells were excluded from the analysis by propidiumiodide staining. To measure the relative amount of FITC-avidin D bound, the mean fluorescence of 5000 stained cells was determined. Background fluorescence, measured in the absence of peptide, was subtracted. Background fluorescence varied between 8 to 15 (10) in all binding experiments.

Results: binding to HLA-DR17 (Fig.1) was analyzed by incubating EBV-BLCL homozygous for DR17 with the N-terminal biotinylated peptides. As shown in Fig.1, substitutions were allowed for all except two residues: substitution at position 5 (I) was only allowed when the amino acid L, which is comparable to the original amino acid I in hydrophobicity and size, was substituted. At position 8 (D) all substitutions reduced binding dramatically. Binding was not decreased by substitutions at any other position, a result which is in agreement with other findings about interactions between peptides and class II molecules usually tolerating 80-90% of single amino acid substitutions in the peptide (8). None of the peptides bound to the class II negative B cell line RJ2.2.5 (21) (data not shown). Thus residues 5 (I) and 8 (D) are important for binding to DR17 molecules.

T cell proliferation assays

Proliferation was assayed by mixing 10⁴ T cells, irradiated DR-matched allogeneic PBMC (5 x 10⁴/well) and Ag (final concentration 5 μ g/ml, 0.5 μ g/ml and 0.05 μ g/ml). After 66 h of culture 1 μ Ci [³H]thymidine was added to each well and 18 h later cells were collected on glass fiber filter strips and the radioactivity

incorporated into the DNA was determined by liquid scintillation counting. Both biotinylated and unbiotinylated were tested for T cell proliferation using the DR3-restricted clones CAAp15 1-1, Rp15 1-1, R1F9 and DAAp15 1-1.

5 Results: to identify the amino acid residues in hsp65 p4-15 that are critical in the formation of the determinant recognized by the HLA-DR3 restricted T cell clones, we tested the ability of the substitution analogs to stimulate the T cell clones CAAp15 1-1, Rp15 1-1, R1F9 and DAAp15 1-1. The peptides were tested at
10 concentrations of 0.05, 0.5 and 5µg/ml. The results obtained are summarized in Table I. Whereas substitutions at positions 4 and 10-12 only affected the proliferation of some clones, residues 5 (I), 6 (A), 7 (Y), 8 (D) and 9 (E) were all equally important for the four clones, as they allowed almost no substitutions.
15 Since residues 5 and 8 were already assigned as DR-contacting residues, we cannot draw a conclusion whether they are also epitope residues. However, residues 6, 7 and 9 are clearly epitope residues for all p4-15 reactive T cell clones tested.

Alignment of other DR3-restricted epitopes of hsp70, hsp18
20 and the 30/31kD protein of *M. leprae* with hsp65 p3-13 showed that all peptides, except the second 30/31kD epitope, contained a large hydrophobic (I, L, V) amino acid at position n followed by a negatively charged (D, E) or polar (Q) amino acid at position n+3. Furthermore, we compared the amino acid sequence of the DR52a-
25 restricted epitope TT 1273-1284 (25) and p3-13 of a second 65kD GroEL protein of *M. leprae* (26) which binds to DR17 (Geluk et al., unpublished results) to that of hsp65 p3-13. Though the former peptide is not DR3- but DR52a-restricted, we included this peptide in our analysis as the proposed peptide binding residues of DR17
30 are preserved in the DR52a molecule (10). This comparison showed that these peptides contained the same amino acids at position n (I) and n+3 (D) (Table I).

Competition experiments

35 Inhibition of activation of the DR3-restricted, 65kD reactive T cell clones CAAp15 1-1 and Rp15 1-1 and of the DR3-restricted,

30/31kD (19) reactive T cell clones L10B4 and L10C11 was studied in T cell proliferation assays by mixing 10^4 T cells, irradiated DR-matched allogeneic PBMC (5×10^4 /well), stimulator peptide and competitor peptide (final concentration 10-, 100-, 1000- and 10000- fold excess relative to the stimulator peptide). As the stimulator peptide for the 65kD reactive clones, p4-15 (final concentration 1ng/ml) was used and for the 30/31kD reactive clones the DR3-restricted epitope (final concentration 0.2µg/ml) of the 30/31kD protein (Thole et al. in preparation). The inhibition experiment for the DR2-restricted T cell clone R2F10 and the DR1-restricted T cell clone N3A9, controls for allele specific inhibition, were performed as described above using hsp65 p418-427 (LQAAPALDKL) (20) or hsp65 p412-425 (GGGVTLQLQAAPALD) respectively, as stimulator peptides (final concentration 1ng/ml). Toxicity of competitor peptides, for either T cells or APCs, was checked by mixing either T cells (10^4 /well) and 10% IL2 (Lymphocult-T, Biotest, Frankfurt/M., FRG) or PBMC (5×10^4 /well) and 0.5% PHA (Wellcome Diagnostics, Dartford, UK) with competitor peptide (final concentration 10µg/ml).

Results: single amino acid substitution analogs that completely lacked T cell stimulatory potency for all four T cell clones but still bound to DR17 were tested for their ability to inhibit the response of the p3-13 reactive T cell clones CAAP15 1-1 and Rp15 1-1 to p4-15. The results of these competition experiments are shown in Fig.2A. Peptides substituted at position 6 (A→H, A→Q), position 7 (Y→S, Y→R, Y→P) and position 9 (E→D) were indeed able to inhibit the response induced by p4-15, though the potency to inhibit proliferation varied between the peptides.

As expected, the peptide containing the substitution 8 (D→P) did not compete at all. To exclude the possibility that the non-responsiveness to the competitor peptides for the DR3-restricted clones was due to toxicity of those peptides for either the T cells or the APCs used in the competition experiments, we tested the influence of these peptides on both the IL-2-dependent activation of the DR3-restricted T cells and the PHA-induced proliferation of the APCs. The presence of the competitor peptides

did not result in reduction of proliferation of the T cells nor did it disturb the activation of APCs by PHA (data not shown).

As a control for the peptide specificity of inhibition we tested the hsp65 418-427 peptide in competition experiments. This peptide represents the sequence of a DR2-restricted epitope on this protein and does not bind to DR17 (data not shown). As expected, coincubation of p418-427 and p4-15 did not reduce the proliferation of the DR3-restricted clones (Fig.2A).

To see whether the inhibition of DR3-restricted T cell activation not only is allele specific but might also be dependent on the nature of the stimulator peptide and/or tested T cell clones, we tested the potency of the six competitor peptides to inhibit the activation of two other DR3-restricted, mycobacterial 30/31kD reactive T cell clones from an unrelated individual (L10B4 and L10C11) that are specific for an epitope on the 30/31kD protein (Thole et al. in preparation). As shown in Fig.2B, all six DR3-specific competitor peptides are able to inhibit the response of clones L10B4 and L10C11 to the 30/31kD peptide. This shows that the DR3-specific T cell inhibition by the competitor peptides does not depend on either the stimulated T cell clone or the stimulator peptide.

In order to check the allele specificity of the inhibition, we tested the p4-15 analogs for their ability to inhibit the response of either the DR2-restricted T cell clone R2F10 or the DR1-restricted T cell clone N3A9, which are stimulated by hsp65 p418-427 and p412-425 respectively. None of the 6 peptides (which were all not stimulatory for R2F10 or N3A9) were able to inhibit the proliferative response of R2F10 to p418-427 or p412-425 to any extent (Fig.2C). This indicates that the competitor peptides inhibit the T cell response of CAAP15 1-1 and Rp15 1-1 in an allele specific manner. Furthermore none of the biotinylated analogs of the competitor peptides bind to any other HLA-DR type (data not shown). This further shows that the competitor peptides bind selectively to DR17.

Finally, to examine whether the mere presence of the DR17-binding motif in a peptide would be enough to inhibit the DR3-

restricted T cell responses, we tested the 6 other DR3-restricted epitopes, including the one without the motif (Table I), for their capacity to inhibit the p3-13 induced T cell activation. As shown in Fig.3, none of the other epitopes were able to reduce the response of T cell clone CAAp15 1-1 to p3-13.

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TABLE I

Summary of involvement of hsp65 p4-15 residues in DR17 binding and DR3-restricted T cell activation

Residue	Position	Role in ¹				
		DR17 binding	CAAp15 (Vβ1)	TCR contacting Rp15 (Vβ5.1)	TCR contacting of R1F9 (Vβ5.1)	DAAp15 (Vβ4)
T	4	-	-	+	+	±
I	5	+	?	?	?	?
A	6	-	+	+	+	+
Y	7	-	+	+	+	+
D	8	+	?	?	?	?
E	9	-	+	+	+	+
E	10	-	+	-	+	+
A	11	-	+	-	+	+
R	12	-	+	-	-	+
R	13	-	-	-	-	-
G	14	-	-	-	-	-
L	15	-	-	-	-	-

¹ (+) indicates a critical role for the amino acid in contacting either the TCR or the DR17 molecule; (±) indicates that the residue can be substituted by other amino acids but partly loses its ability to activate T cells; (-) indicates that the amino acid does not contribute to either TCR or MHC binding; (?) indicates probably no critical role for TCR contacting, but because of a positive involvement in MHC binding this could not be definitely interpreted.

TABLE II

Motif alignments of DR17 binding peptides¹

Peptide	Motif positions											
				1	2	3	4					
hsp65 2-13			A	K	T	I	A	Y	D	E	E	A
TT 1273-1284				G	Q	I	G	N	D	P	N	R
2nd 65kD 2-13			S	K	L	I	E	Y	D	E	T	A
hsp70257-269	S	D	K	N	P	L	F	L	D	E	Q	L
hsp18 61-70			V	V	T	V	R	A	E	R	P	G
30/31kD 62-71			M	G	R	S	I	K	V	Q	L	Q
30/31kD 138-146			T	Y	K	W	E	T	F	L	T	

¹ amino acids that are part of the motif are shown in bold; residues in italics indicate the presence of the motif in the reversed fashion; dots indicate the positions of the amino acids whose presence is required to still obtain binding to DR17.

SEQUENCE LISTING

SEQ ID NO:1

TYPE: amino acid

LENGTH: 7 amino acids

ThrIleAlaTyrAspAspGlu
1 5 7

SEQ ID NO:2

TYPE: amino acid

LENGTH: 7 amino acids

ThrIleAlaSerAspGluGlu
1 5 7

SEQ ID NO:3

TYPE: amino acid

LENGTH: 7 amino acids

ThrIleAlaArgAspGluGlu
1 5 7

SEQ ID NO:4

TYPE: amino acid

LENGTH: 7 amino acids

ThrIleAlaProAspGluGlu
1 5 7

SEQ ID NO:5

TYPE: amino acid

LENGTH: 7 amino acids

ThrIleHisTyrAspGluGlu
1 5 7

SEQ ID NO:6

TYPE: amino acid

LENGTH: 7 amino acids

ThrIleGlnTyrAspGluGlu
1 5 7

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CLAIMS

1. A peptide comprising the amino acid sequence

A_{n-1} A_n A_{n+1} A_{n+2} A_{n+3} A_{n+4} A_{n+5}

wherein

A_{n-1} is any amino acid;

5 A_n is I, L or V;

A_{n+1} is A, H or Q;

A_{n+2} is Y, S, R or P;

A_{n+3} is D, E or Q;

A_{n+4} is E or D;

10 A_{n+5} is any amino acid;

with the proviso that said sequence differs by one amino acid substitution from the sequence T I A Y D E E.

2. The peptide of claim 1, wherein A_{n-1} is T.

3. The peptide of claim 1, wherein A_{n+5} is E.

15 4. The peptide of claim 1, wherein A_n is I.

5. The peptide of claim 1, wherein A_{n+3} is D.

6. The peptide of claim 1, wherein

A_{n-1} is T;

A_n is I;

20 A_{n+1} is A, H or Q;

A_{n+2} is Y, S, R or P;

A_{n+3} is D;

A_{n+4} is E or D;

A_{n+5} is E.

25 7. The peptide of claim 1, wherein the amino acid sequence

A_{n-1} A_n A_{n+1} A_{n+2} A_{n+3} A_{n+4} A_{n+5}

is selected from the group consisting of

T I A Y D D E

T I A S D E E

30 T I A R D E E

T I A P D E E

T I H Y D E E

T I Q Y D E E

8. The peptide of claim 1, having a length of from about 7 to about 30 amino acids.
9. The peptide of claim 1, having a length of from about 8 to about 20 amino acids.
- 5 10. The peptide of claim 1, wherein the amino group of the amino-terminal amino acid is modified, or the carboxy group of the carboxy-terminal amino acid is modified, or both.
11. The peptide of claim 1, wherein the amino group of the amino-terminal amino acid is acylated, or the carboxy group of the
- 10 12. The peptide of claim 1 for treating a HLA DR3 related autoimmune disease with a person or mammal suffering from said HLA DR3 related autoimmune disease.
13. A pharmaceutical composition comprising a HLA DR3 blocking
- 15 effective amount of a peptide according to any one of claims 1 to 12 and a pharmaceutically acceptable carrier or diluent.
14. A method of treating a HLA DR3 related autoimmune disease, comprising administering a HLA DR3 blocking effective amount of a
- 20 peptide according to any one of claims 1 to 12 to a person or mammal suffering from said HLA DR3 related autoimmune disease.

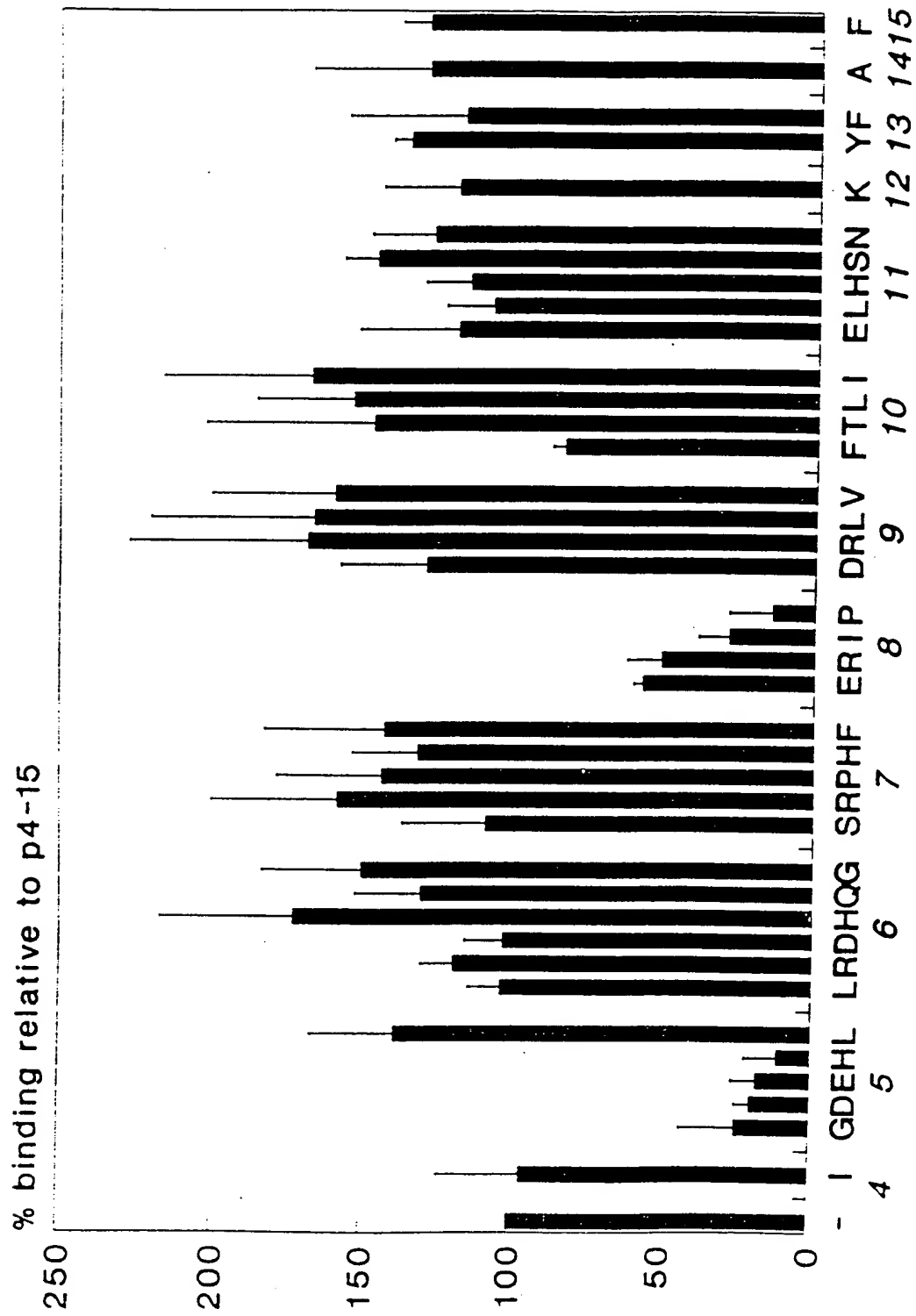


FIG.1

CAAp15 1-1 (DR3)

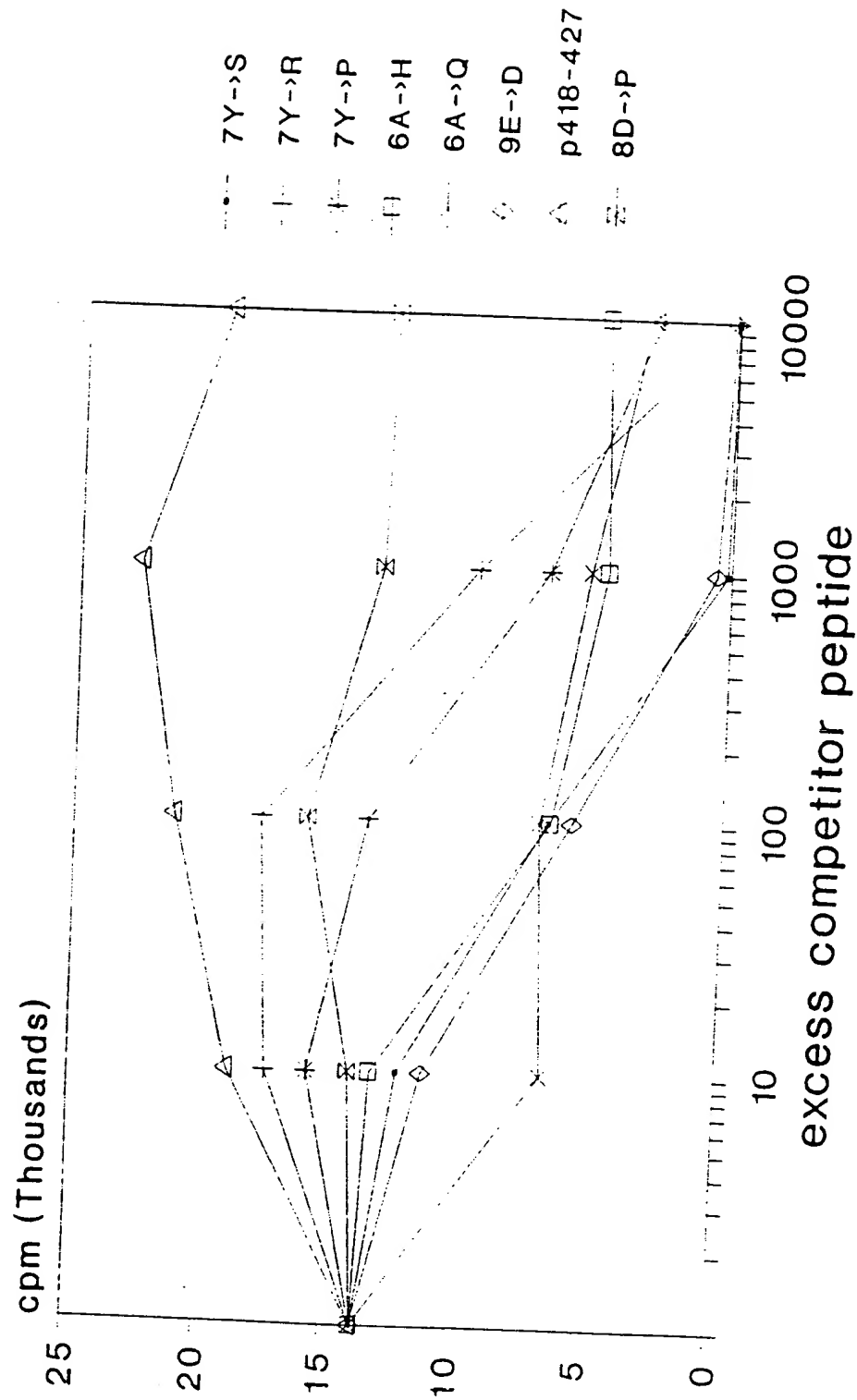


FIG. 2A(1)

Rp15 1-1 (DR3)

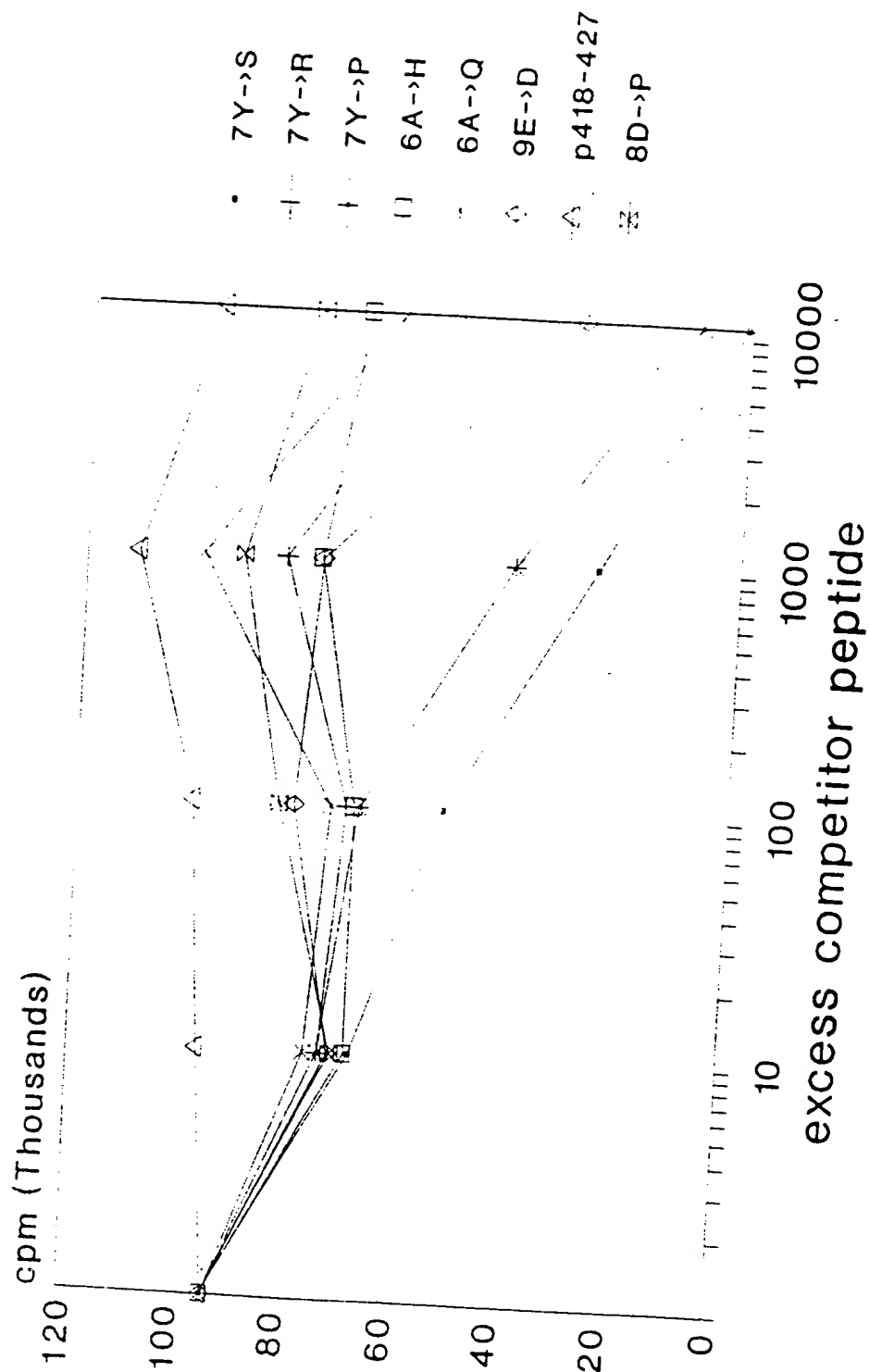


FIG. 2A(2)

L10B4 (DR3)

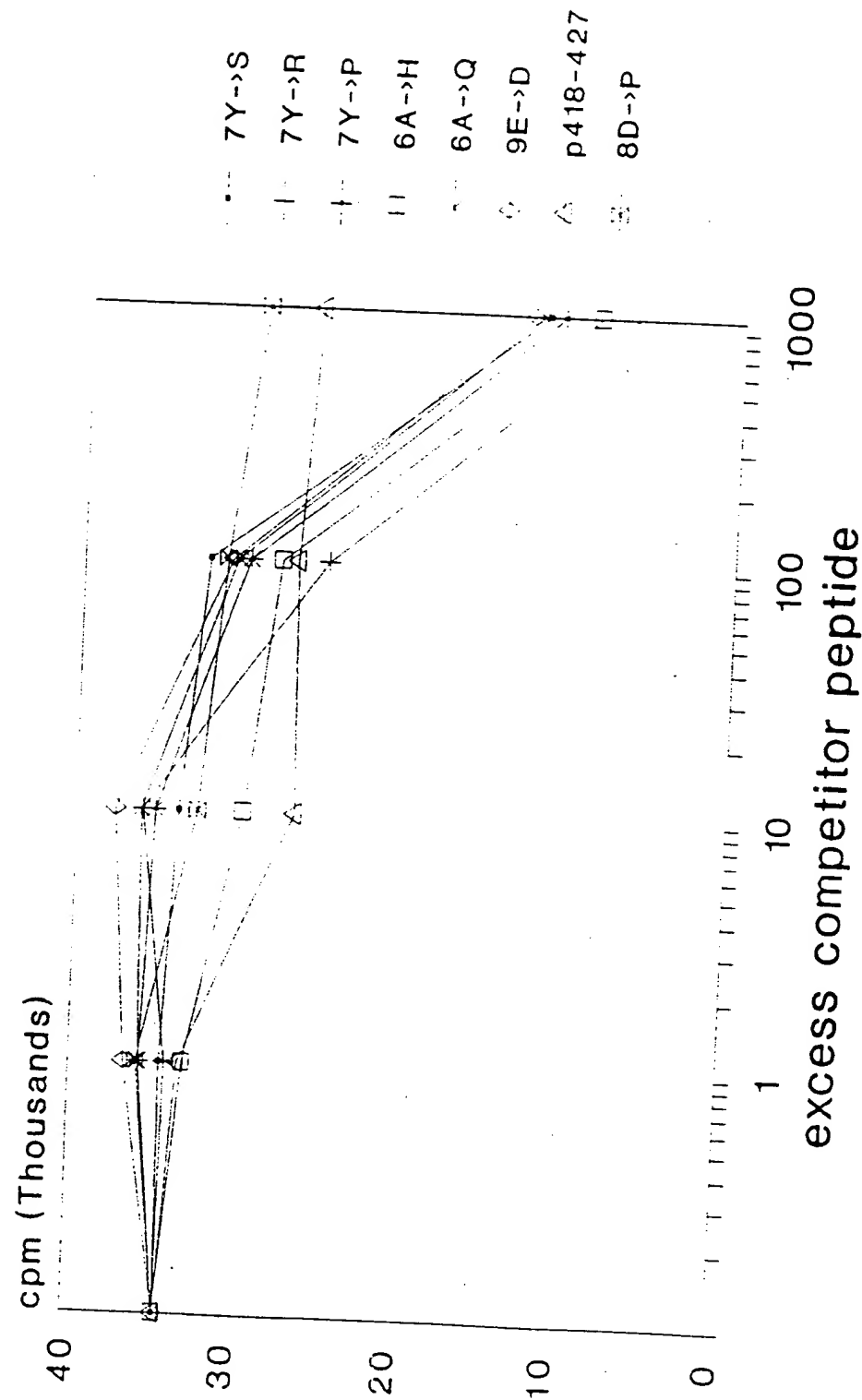


FIG. 2B(1)

L10C11 (DR3)

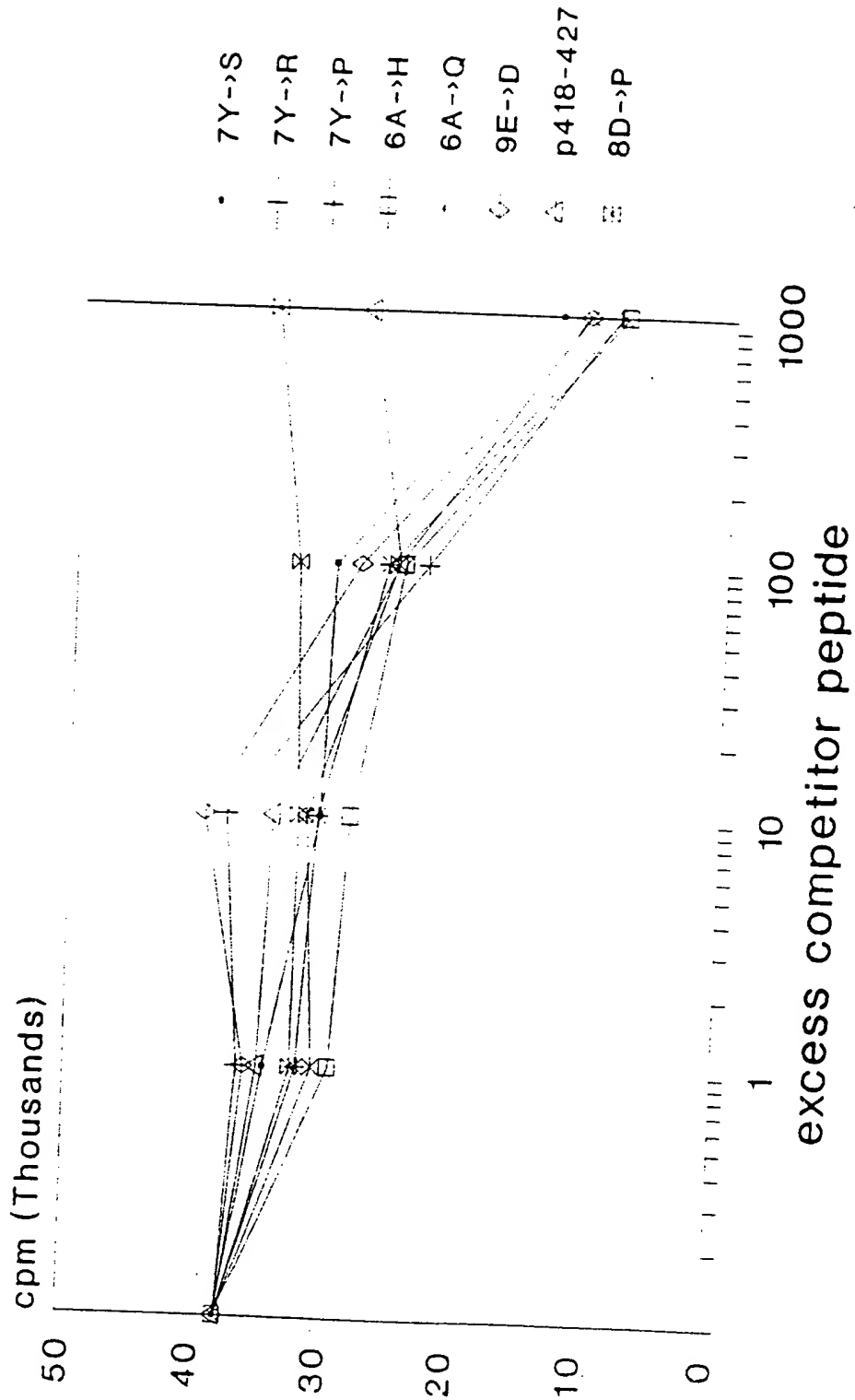


FIG. 2B(2)

N3A9 (DR1)

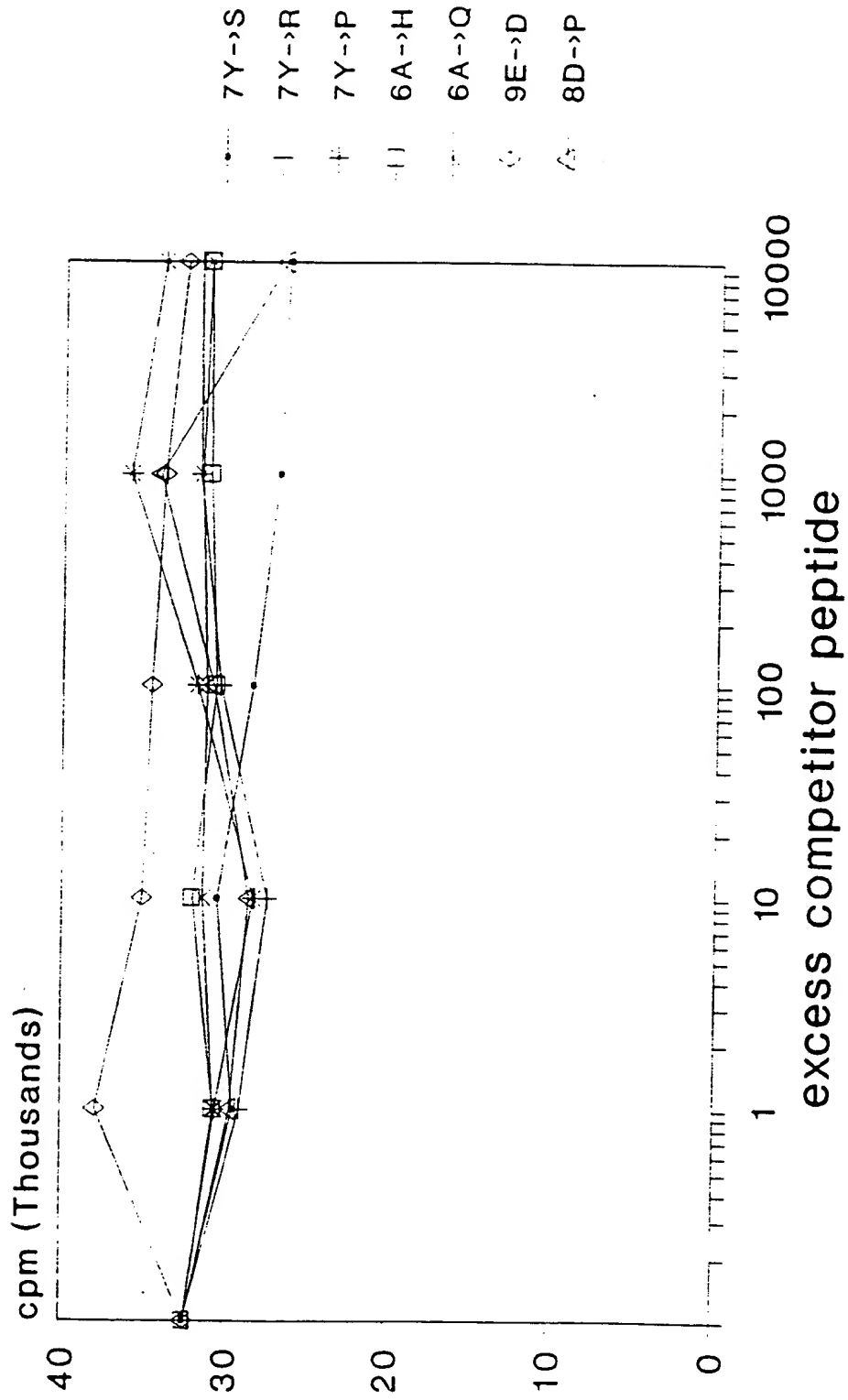


FIG. 2C(1)

R2F10 (DR2)

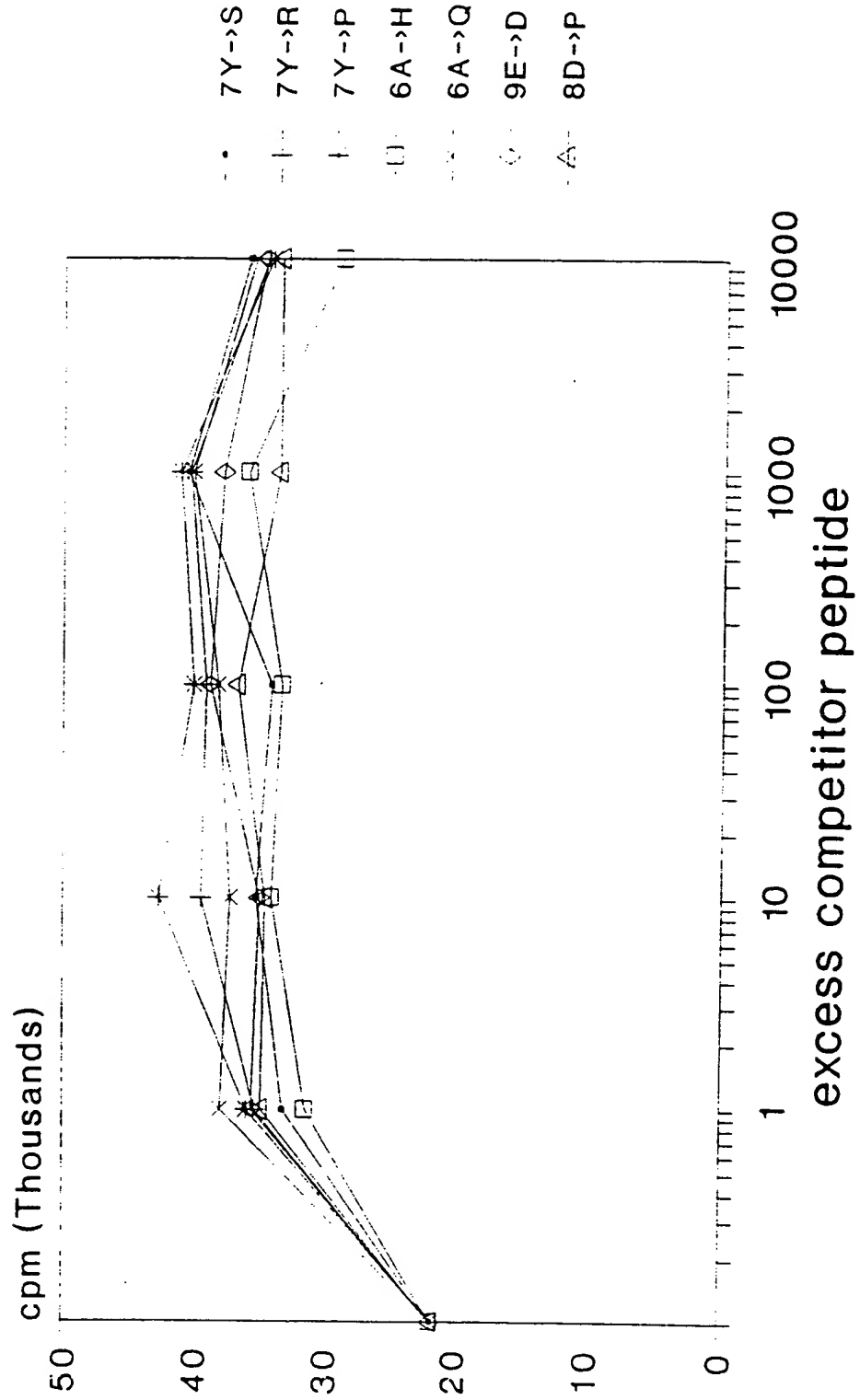


FIG. 2C(2)

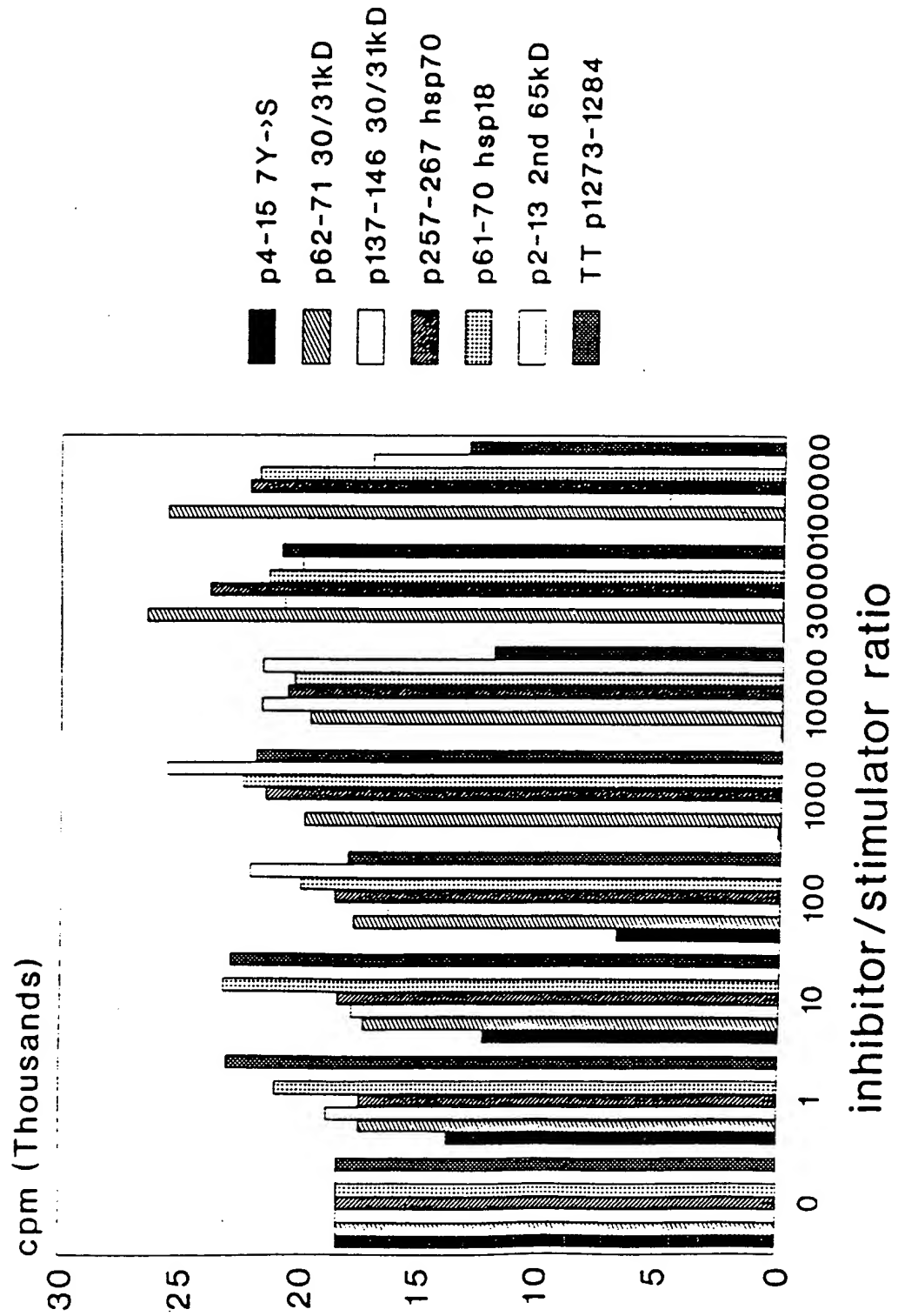


FIG. 3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NUCLEIC ACIDS RESEARCH vol. 15, no. 16, 25 August 1987, ARLINGTON, VIRGINIA US page 6742 A. KONIECZNY 'Nucleotide sequence of lupin leghemoglobin I cDNA' see the whole document ---	1,2,4,5
X	EUROPEAN JOURNAL OF IMMUNOLOGY vol. 22, no. 1, January 1992, WEINHEIM pages 107 - 113 A. GELUK ET AL. 'Binding of a major T cell epitope of mycobacteria to a specific pocket within HLA-DRw17(DR3) molecules' cited in the application see page 108, left column, paragraph 1 see page 108, right column, paragraph 4 * pages 110-112, discussion * ---	1-6,8,9, 13

-/--



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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Date of the actual completion of the international search

23 November 1993

Date of mailing of the international search report

14. 12. 93

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FUHR, C

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0. 262 710 (DE STAAT DER NEDERLANDEN) 6 April 1988 see page 4, line 9 - line 52; claim 1 -----	1,12,13

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 14 is directed to a method of treatment of the human animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

cited in search report

Publication
date

Patent family
member(s)

Publication
date

EP-A-0262710

06-04-88

NL-A- 8602270

05-04-88

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05-04-88

AU-B- 601765

20-09-90

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17-03-88

DE-A- 3781078

17-09-92

JP-A- 63126895

30-05-88

ZA-A- 8706738

14-03-88